

Mechanism of Photosynthetic Carbon Dioxide Uptake by the Red Macroalga, *Chondrus crispus*

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ABSTRACT

The aim of this study was to determine how *Chondrus crispus*, a marine red macroalga, acquires the inorganic carbon (C_i) it utilizes for photosynthetic carbon fixation. Analyses of C_i uptake were done using silicone oil centrifugation (using multicellular fragments of thallus), infrared gas analysis, and gas chromatography. Inhibitors of carbonic anhydrase (CA), the band 3 anion exchange protein and Na^+/K^+ exchange were used in the study. It was found that: (a) *C. crispus* does not accumulate C_i internally above the concentration attainable by diffusion; (b) the initial C_i fixation rate of *C. crispus* fragments saturates at approximately 3 to 4 millimolar C_i ; (c) CA is involved in carbon uptake; its involvement is greatest at high HCO_3^- and low CO_2 concentration, suggesting its participation in the dehydration of HCO_3^- to CO_2 ; (d) *C. crispus* has an intermediate C_i compensation point; and (e) no evidence of any active or facilitated mechanism for the transport of HCO_3^- was detected. These data support the view that photosynthetic C_i uptake does not involve active transport. Rather, CO_2 , derived from HCO_3^- catalyzed by external CA, passively diffuses across the plasma membrane of *C. crispus*. Intracellular CA also enhances the fixation of carbon in *C. crispus*.

Seawater contains approximately 2 mM HCO_3^- and only 10 μ M CO_2 . Thus, it is not surprising that *Chondrus crispus* (4, 9, 10, 28) and other marine macroalgae (2, 13, 14, 25) utilize the HCO_3^- reserves of seawater as a source of photosynthetic carbon, and several of the above authors have suggested that marine macroalgae absorb HCO_3^- . In this study we used the SOC³ technique to determine the mechanism by which *C. crispus* utilizes HCO_3^- . It is the only technique whereby a measurement can be made of the intracellular C_i concentration of algal cells. Previously the technique has been used solely with unicells and plastids, but for our purposes we have found that thallus fragments are equally effective.

As the permeation rate of HCO_3^- through lipid membranes is very slow compared with that of CO_2 (17, 31), some

mechanism to facilitate the uptake or use of HCO_3^- is necessary. Three general mechanisms of HCO_3^- utilization are possible. HCO_3^- may be actively transported across membranes, as occurs in CO_2 -limited cyanobacteria (20). It has been speculated that marine macroalgae actively transport HCO_3^- across their plasma or chloroplastic membranes. This suggestion is based on the ability of macroalgae to photosynthesize at pH values above 8.0 (2, 4, 9, 10, 25), and on their C_4 -like photosynthetic physiology, e.g. low photorespiratory rates (2, 4, 8, 11, 13), low C_i compensation points (4, 12, 13), and intermediate K_m (CO_2) Rubisco values (13). In one study (14), it was shown that several marine macroalgae actively transport C_i for photosynthesis, although no mechanism was adduced. An external CA was not detected in these algae, but their photosynthetic rates were greater than the uncatalyzed rate of CO_2 production from the HCO_3^- in the incubation medium (14).

In a second mechanism of HCO_3^- utilization, the diffusion of HCO_3^- into cells is facilitated by membrane-associated proteins. The band 3 anion exchange protein is the most common HCO_3^- transporting protein in biological systems (27). It has been isolated from corn and oat root (29). In some instances anion transport by the band 3 protein is an active process, presumably utilizing energy from Na^+ gradients across the plasma membrane. The use of band 3 anions exchange protein inhibitors, SITS and DIDS, and Na^+ -free media, have proven useful in characterizing C_i transport in zoological systems (27).

In the third possible mechanism, HCO_3^- simply acts as an external reservoir for CO_2 production and CO_2 is the C_i species that is absorbed. CO_2 diffusing into cells keeps pools of the C_i species at the outer membrane surface in a state of disequilibrium. Therefore, HCO_3^- in the unstirred layer surrounding the algal thallus is constantly converted to CO_2 , according to the physical conditions and the respective C_i equilibrium constants. The rate of CO_2 uptake is enhanced by the codiffusion of HCO_3^- and CO_2 through the unstirred layer surrounding the cell (17, 18). Further, CA located in the unstirred layer or associated with the plasma membrane increases the CO_2 flux rate across the membrane by 2 to 5 times (17, 33). *Chara* (23) and some unicellular green algae (22) have been reported to use this mechanism of C_i uptake.

In this study we show that *C. crispus* does not acquire its photosynthetic C_i through active or anion transport mechanisms. Rather, CO_2 passively diffuses across the plasma membrane. The process is facilitated by external CA and enhanced by the HCO_3^- reservoir in the unstirred layer around the thallus. In addition, intracellular CA enhances carbon fixation in *C. crispus*.

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³ Abbreviations: SOC, silicone oil centrifugation; ASW, artificial seawater; C_i , inorganic carbon; CA, carbonic anhydrase (EC 4.2.1.1.); DIDS, 4,4'-diisothiocyano-2,2'-disulfonate; DMO, 5,5-dimethylloxazolidine-2,4-dione; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39.); SIS, sorbitol impermeable space; SITS, 4-acetamido-4'-isothiocyano-2,2'-stilbene disulfonate; SWM, enriched seawater medium; WA, Wilbur-Anderson unit of CA activity; IRGA, infrared gas analyzer.

MATERIALS AND METHODS

Plant Material

Chondrus crispus Stackhouse plants used in this study were derived from a laboratory grown unialgal male/female BH stock or were subsampled from a male clone 'T4' tank culture. The plants were kindly supplied by Drs. J. McLachlan and J. Craigie of the Atlantic Research Laboratory of the National Research Council, Halifax, N.S., Canada.

Silicone Oil Centrifugation

The SOC technique was used to determine the change in internal C_i concentration and initial rate of carbon fixation in *C. crispus* fragments as affected by external C_i concentration. It is the only technique whereby a direct comparison can be made between the intra- and extracellular C_i levels of algal cells (1).

Thallus fragments were prepared by finely chopping *C. crispus* thalli over ice with a razor blade. Fragments that remained suspended in medium for 1 min were selected for the experiments, as they behaved like unicells by remaining suspended in medium for the 30 s incubation. Fragments consisted of aggregates of 50 to 200 pigmented medullary cells that were photosynthetically active. Incubations were done in 500 μ L microfuge tubes (Eppendorf) containing, from bottom to top, 20 μ L of killing solution, about 250 μ L of silicone oil, and 150 μ L of incubation medium. The killing solution was composed of 2.5 M glycine buffer containing 0.75% SDS (pH 9.5). The silicone oil layer was a 5:1 mixture of 550:200 silicone fluids (Dow Corning). The oil density must be carefully adjusted because of the very small difference between the densities of cells and the seawater incubation medium.

The incubation medium was composed of 75 μ L of C_i -free algal suspension, 25 μ L of C_i -free treatment solution, and 50 μ L of $\text{NaH}^{14}\text{CO}_3$ (Amersham, CFA.3 diluted to 10 mCi/mmol) in SWM 'C' containing 25 mM Hepes (pH 7.5). Treatment solutions, either control, acetazolamide (Sigma) or ethoxzolamide, were added to the algal suspensions just prior to the incubations. (Ethoxzolamide was synthesized as described in Anon, *Chemical Abstracts*, 1958, Vol 52: 20212 and chemically characterized by melting point, TLC and NMR analyses.) The components of the incubation medium were made from C_i -free stock solutions bubbled with N_2 for 1 h and held *in vacuo* for 0.5 h. All solutions were stored under a stream of N_2 and the algal suspensions were kept in sealed microfuge tubes on ice until used.

The algal suspensions were held under a saturating irradiance of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (4) from a 300 W Sylvania photoflood bulb at 15°C for 30 min prior to incubation to reduce residual C_i in the cell suspension. Incubation, irradiated at 750 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, was initiated by injecting the $\text{NaH}^{14}\text{CO}_3$ solution, and terminated after 30 s by centrifugation for 5 s in an Eppendorf Microfuge 5415. Preliminary studies showed that the rate of ^{14}C -fixation was maximum by 30 s and remained linear for some time thereafter; showing that internal pools were saturated by 30 s.

Following the incubation, 10 μ L of the suspension medium was used to determine the external C_i concentration. After freezing in liquid N_2 , the bottom layer of the tube was cut off and placed in 500 μ L of 0.1 N NaOH. Two 200 μ L samples

were taken; one used to determine the total DPM ($^{14}\text{C}_i$ plus ^{14}C -fixed) and the other to determine the ^{14}C -fixed DPM. The suspension medium and the total ^{14}C samples were mixed with 3 mL of Scintiverse II (Fisher) premixed with 0.2 mL of 0.1 N NaOH, whereas the ^{14}C -fixed samples were mixed with 0.2 mL of 1 N HCl, evaporated to dryness and mixed with 3 mL of Scintiverse II. The samples were counted with an LKB Rack Beta scintillation counter using an external standard for quench correction.

Sorbitol-Impermeable Space

A measure of the internal cell volume of the fragments was needed to calculate the intracellular C_i concentration and to provide a standard reference base for the carbon fixation measurements. The internal cell volume, represented by the SIS, was determined using separate $^3\text{H}_2\text{O}$ (Amersham, TRS.8, diluted to 0.2 mCi/mL) and [^{14}C]sorbitol (Amersham, CFB.28, 304 mCi/mmol) incubations. The fragments were found to be not perceptibly permeable to sorbitol in 30 s, whereas they reached saturation with $^3\text{H}_2\text{O}$ within 30 s, so this period of time was chosen for the incubations.

In both incubations, 75 μ L of cell suspension was mixed with 75 μ L of isotope solution for 30 s, using about 0.25 μ L of $^3\text{H}_2\text{O}$ and 1.00 μ L of [^{14}C]sorbitol per incubation. The samples were processed as for the $\text{NaH}^{14}\text{CO}_3$ incubations. SIS was determined by calculating the difference between the $^3\text{H}_2\text{O}$ and [^{14}C]sorbitol-impermeable volumes in the killing solution after centrifugation (1).

IR Gas Analysis

The steady-state C_i uptake rate of *C. crispus* plants was determined using the air-suspension differential IRGA technique described elsewhere (4, 28). Briefly, about 1.0 g of whole thallus was placed wet, but in air, on an inert support in a thermostatted photosynthesis chamber. Gas passed through the chamber and its CO_2 content was compared with that of the incoming gas using the differential mode of an IRGA (Analytical Development Company, Hoddesdon, England, Mark II).

Prior to the air-suspension incubation, the plants were pretreated in seawater (control) or one of the following treatments. Disulfonated stilbenes, SITS and DIDS (Sigma), were used as inhibitors of the band 3 anion exchange protein, responsible for the facilitated diffusion or active transport of C_i in biological systems (27). The effect of Na^+ on the C_i uptake rate of *C. crispus* was determined by pretreating plants in seawater, Na^+ -free ASW (NaCl was replaced with choline-Cl), ASW containing oubain (an inhibitor of Na^+/K^+ exchange), and Na^+ -free ASW containing oubain. The absolute Na^+ level was determined to be 1.95×10^3 ppm in seawater and 1.82 ppm in the choline seawater by atomic absorption spectroscopy. Treatments lasted for 10 min in an ice bath.

Intracellular pH.

Intracellular pH was determined during incubations using the DMO technique (1). Fragments were incubated under light (300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; Sylvania, 300 W photoflood) with 50 μ L of [^{14}C]DMO (Amersham, CFA.575, 50 mCi/mmol)

in SWM 'C' containing 25 mM Hepes at pH 7.50 and 1 mM C_i. Samples were taken during the interval 20 to 30 min after the addition of [¹⁴C]DMO. Preliminary tests showed that accumulation of [¹⁴C]DMO in the fragments was complete within 5 min and DMO content remained constant for up to 45 min. The samples were processed as for the NaH¹⁴CO₃ incubations.

Inorganic Carbon Compensation Point

The C_i compensation point of *C. crispus* was determined using the technique outlined by Birmingham and Colman (6). The experiments were conducted in Dr. Colman's laboratory at York University (Downsview, Ontario). The plants were transported on ice and kept in Instant Ocean at 4°C under low light for 2 to 12 d before use.

The plants were placed in an incubation chamber containing 40 mL of ASW and irradiated with a photon flux density of about 900 μmol·m⁻²·s⁻¹. The medium was stirred, kept at 15°C with a thermostatted circulating water bath and covered with degassed paraffin oil to reduce gas exchange between the atmosphere and the incubation medium. Acetazolamide at 100 μM was added to the incubation medium in some of the tests.

The C_i compensation point of the plant was taken as the point at which the amount of C_i in the medium remained constant for 30 to 60 min. The C_i level in the medium was determined by gas chromatography (6) with a Shimadzu GC-8A. Samples from 0.1 to 0.5 mL were injected into a stripping column containing 0.25 mL of 50% phosphoric acid.

Carbonic Anhydrase Assay

To measure externally located CA, intact *C. crispus* plants were placed in 20 mL of 0.45 μM Millipore-filtered seawater containing 50 mM Tris at pH 8.2 in a 30 mL medium fritted filter funnel. The mixture was bubbled with a gas mixture containing 5% CO₂ and 95% N₂ (Analyzed Gases, Liquid Carbonic Ltd., Toronto) at 100 mL·min⁻¹ and the time required for the pH to drop from 8.100 to 8.000 was recorded (Fisher, Accumet 825 MP pH meter and 13-639-272 pH electrode). Algal CA activities were derived by referring the time required by the plants, weighing between 0.21 and 1.53 g, to reduce the pH to a standard curve prepared with purified CA (C7500, Sigma). Temperature was kept at 8°C by a thermostatted circulating water bath. Trials with plants or purified CA in which 100 μM acetazolamide was present yielded results equivalent to the control situation in which neither CA nor plants were added.

RESULTS

Effect of CA Inhibitors

The internal C_i concentration of *C. crispus* fragments did not generally exceed the C_i concentration of their suspension medium (Fig. 1). Also, the CA inhibitors, acetazolamide and ethoxzolamide, did not affect the level of C_i accumulation by fragments.

The initial C_i fixation rates, as determined using SOC, for *C. crispus* fragments and fragments treated with acetazolamide or ethoxzolamide are shown in Figure 2. Fragments

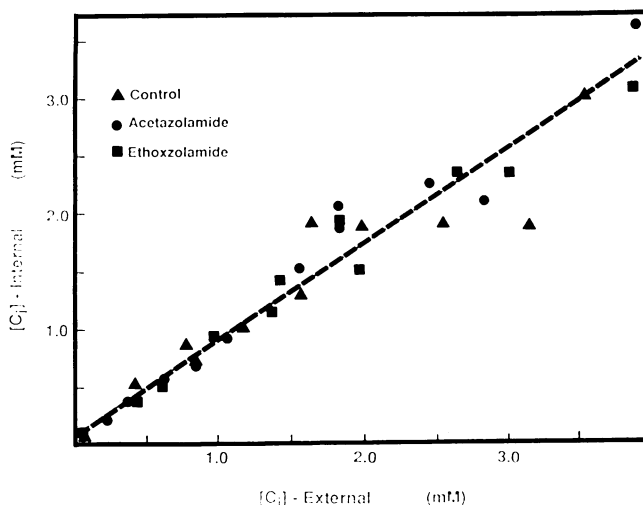


Figure 1. Intracellular carbon level of *C. crispus* fragments as affected by C_i level and the CA inhibitors, acetazolamide and ethoxzolamide (at 160 μM; 750 μmol·m⁻²·s⁻¹; 15°C; SIS, 0.30 μL; intracellular pH, 7.52). Datum points represent single measurements, regression line for the combined data is $y = 0.82x + 0.10$, $r = 0.96$, $n = 30$ (significant at the 95% confidence level).

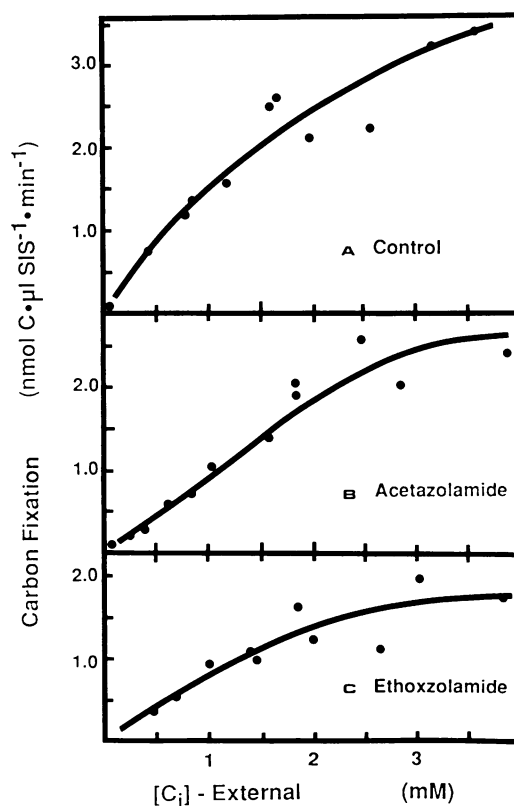


Figure 2. Rate of initial photosynthetic carbon fixation by *C. crispus* fragments (750 μmol·m⁻²·s⁻¹; 15°C; SIS, 0.30 μL; intracellular pH, 7.52) as affected by external C_i level and CA inhibitors (160 μM). A, Control; B, acetazolamide; C, ethoxzolamide. Datum points represent single measurements, lines fitted by eye.

Table I. Amount of Carbon Fixed After 30 s, as nmol C, by *C. crispus* Fragments ($[C_i]$, 0.20 mM; $750 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$; 15°C) as Affected by Acetazolamide (250 μM) at pH Values of 7.0, 8.0, and 9.0

The data represent the mean and their standard deviations, $n = 9$.

Treatment	pH		
	7.0	8.0	9.0
Control	0.117 ± 0.024	0.050 ± 0.015	0.011 ± 0.002
Acetazol	0.102 ± 0.015	0.031 ± 0.006	0.007 ± 0.001
% Inhibition	13%	38%	36%

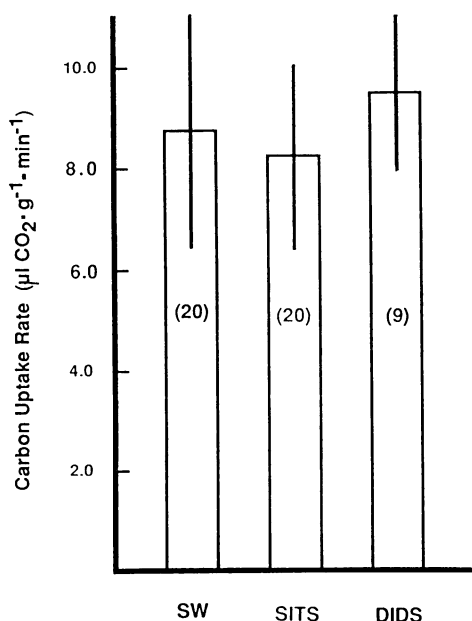


Figure 3. Rate of steady state photosynthetic carbon uptake by *C. crispus* plants ($[CO_2]$ in N_2 , $1000 \mu\text{L} \cdot \text{L}^{-1}$; $400 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$; 12°C) following a 10 min treatment in seawater or seawater containing SITS (1 mM) or DIDS (1 mM). Vertical bars represent the 95% confidence intervals of the means; numbers in parentheses represent n .

had C_i fixation rates of about $3.5 \text{ nmol C} \cdot \mu\text{L SIS}^{-1} \cdot \text{min}^{-1}$, and this rate was attained at external C_i concentrations of about 3 to 4 mM. Acetazolamide and ethoxzolamide inhibited C_i fixation by about 25 and 50%, respectively.

Effect of External pH

The initial C_i fixation rate of fragments was highest when incubated at pH 7 and lowest when incubated at pH 9 (Table I). The percentage inhibition of C_i fixation by acetazolamide was lowest at pH 7 and considerably higher at pH values of 8 and 9. The change in external pH altered, among other things, the proportion of C_i as CO_2 . Before the fragments were added to the microfuge tubes to start the incubations, the equilibrium CO_2 concentration in the medium were 20.4, 2.1, and 0.1 μM CO_2 at the pH of 7, 8, and 9, respectively (the total C_i concentration was kept constant at 200 μM)

Active and Facilitated HCO_3^- Transport.

Inhibitors of the band 3 anion exchange protein (SITS and DIDS; Fig. 3), and the Na^+/K^+ exchange porter (ouabain) and

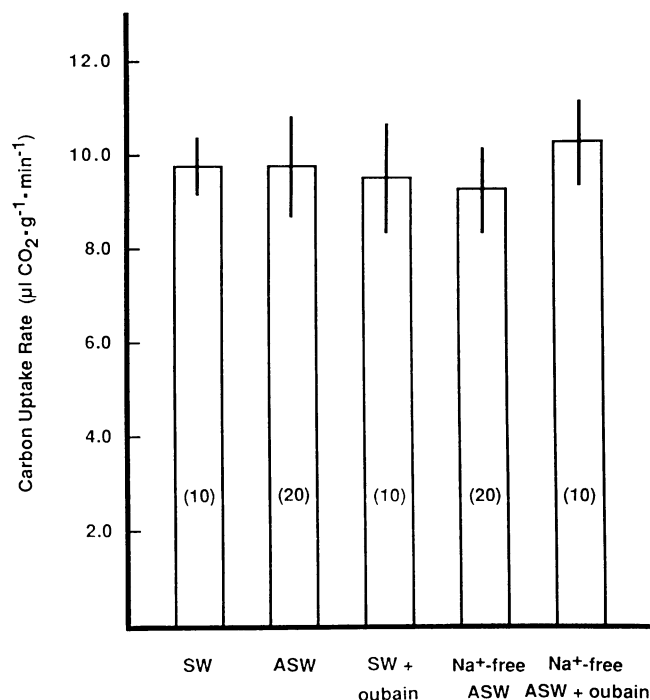


Figure 4. Rate of steady state photosynthetic carbon uptake by *C. crispus* plants ($[CO_2]$ in N_2 , $1000 \mu\text{L} \cdot \text{L}^{-1}$; $400 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$; 12°C) following a 10 min treatment in either seawater (SW), artificial seawater (ASW), ASW containing ouabain (1 mM), Na^+ -free ASW (NaCl replaced by choline-Cl), or Na^+ -free ASW containing ouabain. Vertical bars represent the 95% confidence intervals of the mean; numbers in parentheses represent n .

Table II. Inorganic Carbon Compensation Point (μM) of *C. crispus* Plants ($900 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$; 15°C) at pH values of 7.0 and 8.0 and Treated with Acetazolamide (Acet; 100 μM)

Data are means with their standard deviations given in parentheses.

	pH			
	7		8	
	Control $n = 6$	Acet $n = 6$	Control $n = 8$	Acet $n = 6$
C_i compensation point, μM	145 (28)	241 (53)	147 (47)	292 (114)
Increase	1.66		1.99	

incubation in Na^+ -free SWM (Fig. 4) did not affect the C_i uptake rate of *C. crispus* plants as measured by IRGA analysis. These data indicate that the band 3 anion exchange protein and Na^+ ion-dependent pumps are not involved in the transport of C_i in *C. crispus*.

CA Activity

Intact *C. crispus* plants had a CA activity, using bovine CA as a standard, of $145 \pm 18 \mu\text{g} \cdot \text{g alga}^{-1}$ (SD, $n = 10$). This corresponds to a mean CA activity of 0.36 WA units per g of alga.

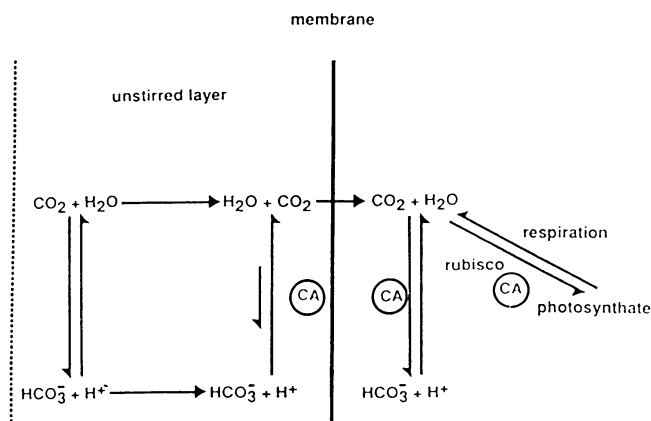


Figure 5. Proposed model of C_i uptake by *C. crispus* tissues. CO_2 diffuses across the plasma membrane of *C. crispus*. This process is facilitated by the presence of externally located CA and is enhanced by the large reservoir of HCO_3^- in the unstirred layer surrounding the thallus. The external CA may be membrane-bound or located in the extracellular matrix of the *C. crispus* thallus; in any event its active site is accessible to the external environment. Internally located CA also enhances the fixation of CO_2 .

Intracellular pH

C. crispus fragments incubated in medium buffered at pH 7.50 had an intracellular H^+ concentration of $2.65 \times 10^{-8} \pm 1.50 \times 10^{-9}$ M (SD, $n = 10$). This corresponds to an intracellular pH of 7.58, the measurements ranged from 7.51 to 7.60.

Inorganic Carbon Compensation Point

The C_i compensation point of *C. crispus* plants was about $145 \mu M$ C_i (Table II). The pH of the incubation medium did not affect this value. However, when the plants were treated with acetazolamide the compensation point was increased by a factor of 1.7 at pH 7.0 and 2.0 at pH 8.0.

DISCUSSION

Carbonic Anhydrase Activity

In this study, intact *C. crispus* plants had measured CA activities of 0.36 WA units $\cdot g$ alga $^{-1}$. Since the plants were intact in the assay, this indicates that the active site of CA was exposed to the external environment, either membrane-associated or within the extracellular matrix of the thallus. That externally located CA is present in *C. crispus* is further supported by the previously reported difference in the affects of acetazolamide, which permeates cell membranes, and dextran-bound acetazolamide, which does not (28).

Smith and Bidwell (28) reported that *C. crispus* crude homogenates had total CA activities of about 9 WA units $\cdot g$ alga $^{-1}$, a value that lies within the range of CA activities reported for a variety of marine macroalgae (16). Therefore, the external CA activity measured in *C. crispus* plants in the present study represents about 4% of the total, a value comparable to that found in some unicellular green algae (30) and endothelial mammalian lung tissue (33). Other microalgae (e.g. *Dunaliella* and *Chlamydomonas*) possess CA mainly at their outer membrane surface (22, 30).

In contrast to data reported in this study for *C. crispus*, external CA has not been detected in several other red and brown algae (14). The technique that was used to test for an externally located CA was somewhat similar to the technique we used, and both techniques are able to detect purified CA (Sigma C7500) at levels as low as 0.5 ng/mL of solution. An external CA is apparently not universal among marine macroalgae.

Internal Inorganic Carbon Levels

When *C. crispus* plants are incubated at pH levels of 7.50 their intracellular pH, as determined by the DMO technique, is about 7.58. From this calculation and data showing that *C. crispus* fragments do not accumulate C_i above the concentration in their incubation medium (Fig. 1), it can be concluded that these plants acquire their photosynthetic C_i through a diffusive rather than active mechanism. This is in contrast to cyanobacteria and green algae that can accumulate C_i to a concentration of 1000 and 40 times, respectively, that found in their environment (1, 19).

Acetazolamide and ethoxzolamide did not reduce the internal C_i concentration of fragments in the SOC studies (Fig. 1), that is, the internal C_i concentration was not dependent on an externally located CA. However, in this experiment the uncatalyzed supply rate of CO_2 , 0.98 nmol $\cdot s^{-1}$ at 2 mM external C_i (calculated by the method of Miller and Colman [21]), far exceeded the combined measured rates of C_i uptake and fixation by *C. crispus* fragments, 0.03 nmol $\cdot s^{-1}$. In other words, the conditions in this experiment were such that C_i accumulation was already maximal and thus would be unaffected by externally located CA.

Initial Carbon Fixation

Using the SOC technique, it was determined that the maximum initial C fixation rate of *C. crispus* fragments was about 3.5 nmol C $\cdot \mu L$ SIS $^{-1} \cdot min^{-1}$ (Fig. 2). This is comparable to the C fixation rate reported for 'high CO_2 ' grown *Chlamydomonas* cells, which do not concentrate C_i (Fig. 1 of Badger *et al.* [1]).

The C fixation rates of *C. crispus* fragments were maximum at about 3 to 4 mM C_i (Fig. 2). This is somewhat higher than the C_i saturation concentrations of 2 to 3 mM C_i for whole plants as measured with the IRGA technique (28). However, whole plants were in steady state during IRGA measurements whereas fragments had been depleted of their internal C_i reserves prior to the short SOC incubations. For rapid C fixation, cells require internal C_i to activate Rubisco and as a substrate for fixation (24). Building up internal C_i , already present during steady state photosynthesis, may result in the higher C_i saturation level of *C. crispus* fragments using the SOC as compared to the IRGA technique (24).

C. crispus and other marine macroalgae saturate at C_i levels near 2 mM or higher (this study; also see 2, 4, 8, 25, 28). In contrast, cyanobacteria and green algae, which actively accumulate C_i , are saturated at levels less than 0.1 mM C_i (1, 19). This phenomenon suggests that *C. crispus* plants (and presumably other marine macroalgae as well) do not actively accumulate C_i , but acquire C_i through diffusive mechanisms.

The initial C fixation by *C. crispus* fragments, incubated at

a constant C_i concentration, was high at pH 7.0, intermediate at pH 8.0 and low at pH 9.0 (Table I). Also, acetazolamide inhibited carbon fixation by 13% at pH 7.0 and about 35% at pH values of 8.0 and 9.0. The higher initial C_i fixation rate and lower acetazolamide inhibition at pH 7.0 than at pH 8.0 and 9.0 is proportional to the percentage of C_i as CO_2 at these pH levels (10.22, 1.07, and 0.07%, respectively), and inversely proportional to the percentage of C_i as HCO_3^- . These data indicate that CO_2 is the preferred C_i species being taken up by *C. crispus* fragments, and are consistent with the suggestion that CO_2 diffuses across the plasma membrane of *C. crispus*.

Similarly, the productivity of cultured *C. crispus* plants is greatest when grown at pH values well below 8 (5, 26). In those studies, pH was kept constant with a pH stat controlling CO_2 addition. Although the plants were exposed to different C_i levels (e.g. higher C_i levels at higher pH values) they were all saturated with respect to C_i . These results are comparable to those presented for several other marine macrophytes (7, 15).

In contrast, the photosynthetic rate of *Gracilaria tikvahiae*, measured over a pH range of 7.0 to 9.5 with the respective increase in C_i concentration at high pH, was positively correlated with the estimated HCO_3^- concentration of the incubation medium (4). This was taken to mean that these plants were absorbing HCO_3^- . However, the possibility was not ruled out that *G. tikvahiae* plants acquire their photosynthetic C_i by the diffusion of CO_2 . If they possess an external CA, the greater HCO_3^- content of the incubation medium at alkaline pH values would increase the carbon uptake rate of the alga by acting as a reservoir for CO_2 .

Steady State Inorganic Carbon Uptake

SITS and DIDS treatments did not affect the steady state C_i uptake rate of *C. crispus* plants (Fig. 3). SITS and DIS are potent inhibitors of the band 3 anion exchange protein. Although this protein is common in animal systems (27) and has been detected in corn and oat roots (29), data from this study indicate that it is not involved in the facilitated diffusion or active transport of C_i in *C. crispus*.

Further, C_i uptake is not Na^+ -dependent in *C. crispus*, as incubation in Na^+ -free medium and ouabain, which inhibits Na^+/K^+ exchange thus reducing potential Na^+ gradients, did not affect the carbon uptake rates of the plants (Fig. 4). In contrast, cyanobacteria accumulate C_i , in part, by Na^+ -dependent mechanisms (20).

Inorganic Carbon Compensation Point

C. crispus plants have a C_i compensation point of about 0.15 mM C_i (Table II). This value corresponds to a compensation point of 35 $\mu L \cdot L^{-1} CO_2$, assuming that the C_i system is in equilibrium. This is unlikely to be the case, because the alga is constantly utilizing and releasing C_i and probably has an unstirred layer associated with it. Nonetheless, the CO_2 compensation reported here is comparable to that reported for *Palmaria* by Cook and Colman (13) using the same technique and in the high end of the range of CO_2 compensation points reported for *C. crispus* and other macroalgae using a variety of techniques (4, 8, 12, 13). Because *C. crispus* plants only have slight, if any, photorespiratory activity, as indicated by the lack of an O_2 effect on their carbon uptake

rate, light-respiratory rate, or compensation point (4, 8, 11), it can be assumed that the differences in the compensation points reported in the different studies were due to the different techniques used, perhaps as a result of unstirred layers and nonequilibrium conditions. Nonetheless, the compensation points reported here and by others are generally lower than those of C_3 terrestrial plants (3). The low and intermediate compensation points reported for marine macroalgae have been used as circumstantial evidence indicative of an operational C_i concentrating mechanism.

The C_i compensation points of *C. crispus* plants are the same at pH values of 7.0 and 8.0 (Table II) when, in this case, the absolute CO_2 concentration is greater by an order of magnitude at pH 7 than at pH 8. This indicates that the plants are able to utilize carbon from both CO_2 and HCO_3^- for carbon uptake. However, acetazolamide increased the C_i compensation point of the plants, having a greater effect at pH 8.0 than pH 7.0. These results are consistent with data of Smith and Bidwell (28) which show that acetazolamide inhibits the carbon uptake rate of *C. crispus* plants to a greater extent when incubated at CO_2 -limited as compared to CO_2 -saturated concentrations. Both sets of results indicate that the utilization of HCO_3^- as a source of photosynthetic carbon is dependent on CA in *C. crispus*. This, in turn, indicates that HCO_3^- must be dehydrated to CO_2 by CA before it can be absorbed.

Inorganic Carbon Transport by *C. crispus*

All these data indicate that whereas HCO_3^- provides the main source for photosynthetic carbon, CO_2 , rather than HCO_3^- , is the molecular species absorbed. These ideas are accommodated in Figure 5, which shows a model for C_i uptake in *C. crispus*. In this model, CO_2 uptake is supported by the high concentration of HCO_3^- in seawater, and the conversion of HCO_3^- to CO_2 prior to absorption is facilitated by externally located CA. Intracellular CA is also shown to enhance carbon fixation.

That *C. crispus* does not actively accumulate C_i is supported by two phenomena. First, *C. crispus* fragments do not accumulate C_i above the concentration attainable by diffusion. Second, photosynthesis in *C. crispus* saturates at C_i concentrations equivalent to the natural level of C_i in seawater, namely 2 mM (this study; 8, 28). Thus it is unlikely that there is an active C_i uptake mechanism in *C. crispus*.

The model in Figure 5 indicates that CO_2 , rather than HCO_3^- , is the C_i species that diffuses across the plasma membrane of *C. crispus* cells. Three conclusions of our study support the model. First, the band 3 anion exchange protein and Na^+ do not facilitate C_i transport in *C. crispus*. Second, the initial carbon fixation rate of *C. crispus* is proportional to CO_2 concentration and inversely proportional to HCO_3^- concentration. Third, the utilization of HCO_3^- for carbon uptake in *C. crispus* is dependent on external CA. Given the relative proportions and amounts of CO_2 and HCO_3^- in seawater, the carbon uptake rate of *C. crispus* plants would not likely have been affected by the CA inhibitors if HCO_3^- was the substrate for C_i transport.

The difference in the permeability coefficients of CO_2 and HCO_3^- for lipid membranes and the effect of turbulence on algal photosynthesis further support the conclusion that CO_2 , rather than HCO_3^- , diffuses across *C. crispus* thalli. CO_2

readily diffuses through lipid membranes whereas the permeation of HCO₃⁻ through lipid membranes is slow and considered insignificant (17, 31). The photosynthetic rate of marine macroalgae is dependent on water velocity and the thickness of the unstirred layer surrounding their thalli (32). This result is consistent with *C. crispus*, and perhaps other macroalgae, taking up CO₂ by diffusion, photosynthesis being CO₂-limited when water velocities about the algae are relatively low, creating relatively thick, unstirred layers. The HCO₃⁻ present, even in thick, unstirred layers, should be sufficient to saturate photosynthesis if the algae were taking up HCO₃⁻ (17). It also appears likely that the mechanism would result in a sufficiently high CO₂ to O₂ ratio, or scavenging of CO₂, to result in the low rates of photorespiratory gas exchange rates observed for *C. crispus* (4, 8, 11).

Intracellular CA, which also enhances the carbon fixation rate of *C. crispus* plants, may do so by enhancing the rate that CO₂ is transported to the active site of Rubisco or by helping catalyze carboxylation and the water splitting reaction (22).

The C_i transport system described for *C. crispus* is not universal among the marine macroalgae. Several red and brown algae have been reported to transport HCO₃⁻ actively across their plasma membranes (14). The data supporting this conclusion indicated that the algae studied did not possess external CA that the carbon uptake rates of the algae exceeded the maximum uncatalyzed rate of CO₂ production from HCO₃⁻ reserves in the incubation medium.

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